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Increased Expression of Aphidicolin-Induced Common Fragile Sites in Tourette Syndrome: The Key to Understand the Genetics of Comorbid Phenotypes?

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In a comparison of 80 common aphidicolin-induced fragile sites (FS) between 26 DSM-IV Tourette syndrome (TS) and 24 control individuals, the mean of the summed break frequencies following mild aphidicolin pretreatment was significantly higher in TS individuals than in controls ($P < 0.001$). Other breakpoints encountered during this study, i.e., random breaks, breaks corresponding to rare FS, and breakpoints recorded by others but not listed as common FS according to the Chromosome Coordinating Meeting [1992] were listed as category II breakpoints. By using the most significantly different mean FS breakage figures between TS and control individuals, further stepwise discriminant analysis allowed identification of TS individuals from only a few sites in both the common FS and category II breakpoint groups. Future research needs to focus on confirmation of altered common fragile site expression in association with behavioral variation, whether expression of certain discriminatory sites concurs with specific comorbid disorder expression; the nature of the molecular alterations at these FS and the implications of a genomic instability phenotype for the mapping of a primary TS gene or genes.

KEY WORDS: Tourette syndrome, aphidicolin, common fragile sites, gene mapping, tandem repeat sequences

INTRODUCTION

Tourette syndrome (TS) is a common, heritable tic disorder associated with obsessive compulsive behavior and attention deficit-hyperactivity which are regarded as integral components of the phenotype [Kurlan et al., 1994], while the relationship to a wide range of additional reported comorbid problems [Comings and Comings, 1993] remains controversial. Most research findings indicate that TS is due to the effect of a single major autosomal gene [Baron et al., 1981; Kidd and Pauls, 1982; Comings et al., 1984; Devor 1984; Pauls and Leckman, 1986], which is supported by genealogical research indicating a gene founder effect in South Africa (Torrington and Gericke, unpublished data). Alternatively, the complex spectrum of manifestations suggested to others the possibility of polygenic inheritance [Comings, 1994].

The recent observation of increased chromosomal breakage in TS has been proposed as a basis for the consideration of variable multiple secondary gene involvement at chromosomal fragile sites in complex neurobehavioral disorders [Gericke et al., 1995].

Fragile sites (FS) are nonrandom heritable sites on chromosomes that can be induced to form gaps, breaks, and rearrangements under specific conditions [Jordan et al., 1990]. Both rare and common sites are expressed in culture under conditions which inhibit DNA synthesis. FS may represent "active genomic sites that are vulnerable to physiological and environmental disturbance" [Yunis and Hoffman, 1989]. Because the different classes of FS reveal cross induction [Yunis et al., 1987; Hecht et al., 1988], it was suggested that FS may be indicative of areas of shared molecular homology in the sequence composition of nonrandom chromosomal DNA [Stopera, 1989], and that such sites can be general targets of mutagenic action.

Since aphidicolin-induced common FS have been postulated to be of pathologic importance [Hecht, 1991], we decided to investigate whether increased fragility of these sites are present in TS individuals, and whether any specific aphidicolin-induced breakpoints could be

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demonstrated to be characteristically associated with TS clinical diagnosis.

MATERIAL AND METHODS

Five to ten milliliters of heparinized blood for chromosome culturing was collected from individuals after informed consent was obtained.

Patients

This group of healthy young adult Caucasians included 15 males and 11 females. All of these 26 individuals had mild, though clearly recognisable motor and/or vocal tics, but were moderately to severely handicapped by associated obsessive-compulsive disorder, residual learning problems, or conduct disorders. Nineteen relatives from 6 families were included in this group. They were diagnosed as having TS by means of DSM IV criteria, had positive family histories of a tic disorder in all instances, and were selected according to whether their clinic visits coincided with days that were convenient to the laboratory, whether they gave informed consent to participate in the study and whether matched controls were available. The choice of this age cohort allowed a clear retrospective evaluation of the longitudinal patient histories and permitted exclusion of other potentially confounding psychiatric or neurologic differential diagnoses.

Controls

Twenty-four randomly selected healthy, emotionally stable Caucasian medical student volunteers consisting of 14 males and 10 females. There were no significant differences in the mean age between patients and controls, and in the mean age of males and females within the groups.

Procedure

Coded blood specimens from both patients and controls arrived simultaneously at the laboratory in batches of varying sizes. Patient and control specimens were thus handled together, and culture preparation and analysis of slides were performed by a single investigator who had no knowledge of the clinical status of the individuals being tested.

Cell Cultures

Phytohemagglutinin (PHA) stimulated blood lymphocytes were cultured in RPMI 1640 medium (Highveld) supplemented with 10 percent fetal calf serum (GIBCO) for 72 hours at 37°C. Twenty-four hours prior to harvesting, aphidicolin dissolved in 70% ethanol (MERCK) was added to each 10 ml culture to obtain a final concentration of 0.1 μM. Colcemid (0.2 ml) (SIGMA) was added for the last 35 minutes. Cells were then treated with a hypotonic 0.075 m KCl solution for 12 minutes and fixed in methanol: acetic acid fixative. Slides were made, air dried and GTG banded.

Analysis

Three to four slides from each individual were analysed, and for each patient one hundred complete metaphases were assessed at 350–400 band level. Aberrations (gaps and breaks) were counted as single events at the band(s) involved. In some metaphases, it was not possible to identify the individual chromosomes and chromosome bands because of the extent of breakage. Such pulverized cells were not included in the analysis.

All events on the band were recorded, i.e. chromatid gap, or break, chromosome gap or break, or where an exchange occurred. If the gap or break occurred on both homologues, in the same band, it was counted as two events.

Frequencies of 80 common aphidicolin-inducible FS were evaluated according to criteria formulated by the Chromosome Coordinating Meeting [1992]. (Table I) All other breakpoints, including rare FS, breaks reported by others but not listed by the Chromosome Coordinating Meeting as common FS, and all breakpoints regarded as random, were listed separately. (Table II) These sites included only those breakpoints from the group, which occurred in most of the individuals in low frequencies, or which occurred in only some of the samples, but in higher frequencies, comparable to those of common FS, in both TS and non-TS groups.

RESULTS

The differences in the mean number of aberrations per cell between TS and controls for known aphidicolin-induced common FS, and for breakpoints, not listed as common FS are shown in Tables I and II.

In order to develop a classification function for characterization of TS patients, a stepwise discriminant analysis was utilized. The sample size was not adequate to include all the sites into the analysis. Hence to decide which sites to include, the two groups were compared with respect to the mean percentage of breaks at each site, using the appropriate t-test after comparing the groups for equal variance using Levene's test. Those sites for which the two groups were significantly different ($P < 0.05$) with regard to the mean percentage of breakages, and who additionally displayed mean differences >5% between patients and controls were subsequently included into a further stepwise discriminant analysis.

Standard Aphidicolin-Inducible Sites (Table 1)

From the sites, 1p21, 1q44, 3p14, 6q26, 7p22, 7q31, 7q32, 11p14, 11q14, 16q23, 22q12, Xp22, and Xq22, utilization of stepwise discriminant analysis indicated optimum standardized coefficients for the sites 1q44, 16q23, and 22q12 which were used in the classification function:

$$\text{Tourette: } y = -16.922 + 0.934(1q44) + 0.395(16q23) + 0.321(22q12)$$

$$\text{Controls: } y = -5.085 + 0.295(1q44) + 0.245(16q23) + 0.133(22q12)$$

and which could be simplified to the single function: 0.639(1q44) + 0.150(16q23) + 0.188(22q12). With all controls scoring less than 11.837, this function indicated 100% specificity and 88% sensitivity for these three sites under these study circumstances.

TABLE 1. Mean (SD) Percentage of Common Aphidicolin-Inducible Breaks at Different Sites for TS and Control Individuals

Site	Tourette patients	Controls	*P* values associated with t-test	Site	Tourette patients	Controls	*P* values associated with t-test
1p36	2.69 (1.57)	1.42 (1.25)	0.003	7q21	4.31 (2.15)	2.42 (1.72)	0.001
1p32	5.00 (2.95)	2.83 (1.61)	0.002	7q22	2.85 (1.89)	1.33 (1.24)	0.002
1p31	4.65 (3.15)	2.00 (1.22)	0.001	7q31	12.62 (4.93)	6.83 (2.97)	0.000
1p22	0.77 (1.07)	0.75 (1.03)	0.949	7q32	17.38 (5.26)	9.29 (3.22)	0.000
1p21	14.42 (5.12)	7.83 (3.20)	0.000	7q36	1.35 (1.13)	0.46 (0.59)	0.001
1q21	1.42 (1.33)	0.67 (0.92)	0.025	8q22	5.38 (2.97)	3.29 (1.99)	0.005
1q25	6.19 (3.15)	2.63 (1.79)	0.000	8q24.1	3.08 (2.28)	1.88 (1.73)	0.042
1q31	1.50 (1.24)	0.75 (0.85)	0.016	8q24.-3	2.15 (1.54)	1.13 (0.99)	0.007
1q42	0.73 (1.28)	0.67 (0.96)	0.844	9p21	1.31 (1.38)	0.54 (0.78)	0.019
1q44	9.85 (0.67)	3.96 (2.03)	0.000	9q12	0.85 (0.88)	0.54 (1.18)	0.303
2p24	9.96 (4.27)	6.04 (2.97)	0.001	9q22	3.27 (1.89)	2.08 (1.47)	0.017
2p16	6.77 (2.57)	3.92 (1.95)	0.000	9q32	12.73 (4.88)	8.04 (3.26)	0.000
2p13	6.96 (2.86)	3.50 (1.79)	0.000	10q21	0.73 (0.96)	0.46 (0.78)	0.279
2q21	6.58 (2.91)	2.71 (1.78)	0.000	10q22	3.00 (1.96)	1.58 (1.32)	0.005
2q31	5.96 (4.15)	2.04 (1.70)	0.000	10q25	3.08 (2.33)	1.25 (1.07)	0.001
2q32	7.77 (3.66)	5.79 (2.20)	0.025	10q26	6.50 (2.94)	4.29 (2.89)	0.010
2q33	5.69 (3.21)	2.21 (1.47)	0.000	11p15	3.62 (1.94)	2.42 (1.18)	0.011
2q37	8.12 (3.70)	4.42 (2.70)	0.000	11p14	10.38 (4.01)	5.08 (2.93)	0.000
3p24	7.19 (4.07)	4.67 (2.58)	0.012	11p13	9.27 (3.64)	5.92 (3.60)	0.002
3p14	58.50 (13.64)	38.42 (9.10)	0.000	11q14	10.19 (4.38)	5.75 (2.66)	0.000
3q25	4.00 (2.48)	2.71 (2.81)	0.091	11q23	0.85 (0.83)	0.54 (0.72)	0.175
3q27	3.73 (2.22)	2.25 (1.65)	0.011	12q21	4.00 (2.51)	2.13 (1.60)	0.003
4p16	2.85 (1.85)	2.21 (1.35)	0.173	12q24	2.65 (1.92)	1.92 (1.35)	0.121
4p15	2.92 (1.90)	2.46 (1.64)	0.361	13q13	8.50 (3.71)	4.29 (2.37)	0.000
4q21	3.19 (2.47)	1.96 (1.71)	0.047	13q21	1.35 (1.44)	1.17 (1.37)	0.655
4q31	12.19 (3.90)	8.17 (3.13)	0.000	13q32	2.65 (1.72)	1.21 (1.25)	0.002
5p14	4.00 (2.12)	2.63 (1.95)	0.021	14q23	5.31 (2.98)	2.38 (1.58)	0.000
5p13	2.31 (2.28)	1.29 (1.37)	0.061	14q24	7.77 (3.12)	4.13 (2.35)	0.000
5q15	5.69 (3.11)	3.88 (2.54)	0.029	15q22	1.42 (1.27)	0.96 (0.91)	0.141
5q21	2.54 (1.75)	1.58 (0.83)	0.017	16q22	1.42 (1.36)	1.04 (0.91)	0.254
5q31	2.04 (1.40)	0.96 (0.86)	0.002	16q23	49.62 (10.18)	27.92 (9.49)	0.000
6p25	6.62 (3.58)	3.42 (1.74)	0.000	17q23	2.88 (1.99)	0.63 (0.65)	0.000
6p22	1.08 (1.23)	0.67 (0.92)	0.191	18q12	5.85 (2.65)	3.08 (1.77)	0.000
6q15	1.58 (1.45)	1.46 (1.06)	0.744	18q21	1.65 (1.47)	1.08 (1.25)	0.147
6q21	3.23 (1.75)	2.29 (1.57)	0.052	19q13	1.50 (1.50)	1.17 (1.43)	0.427
6q26	12.15 (4.86)	7.13 (2.91)	0.000	20p12	5.73 (3.21)	3.33 (2.04)	0.003
7p22	9.23 (3.87)	4.13 (2.89)	0.000	22q12	10.48 (4.35)	5.83 (3.38)	0.000
7p14	1.96 (1.48)	0.54 (0.83)	0.000	Xp22	23.00 (8.60)	14.96 (6.84)	0.001
7p13	11.85 (4.22)	6.04 (3.64)	0.000	Xq22	15.46 (6.41)	9.22 (3.68)	0.000
7q11	4.62 (2.14)	3.00 (1.82)	0.006	Xq27	2.27 (1.97)	1.21 (1.28)	0.028

Category II Sites (Table II) (Fig. 1)

It was decided to keep the analyses separate for the two categories of sites as defined in this article. The overall difference in category II sites between TS and control individuals were significant ($P = 0.001$). By including sites 3p26, 3q13, 10p11.2, 12q12–13, 14q12–13, and Xq13, utilization of stepwise discriminant analysis indicated optimum standardized coefficients for the sites 10p11.2, 14q13, and 3q13. Employing these sites in an analysis similar to that performed for the aphidicolin-inducible group, 73.1% of clinically diagnosed TS patients were assigned to the affected group by means of observation of these site abnormalities (sensitivity), as was the case for 79.2% of controls (specificity).

The classification function for category II sites was

$$\text{Tourette } y = -5.810 + 0.888(10p11.2) + 1.184\,(14q12.3) + 0.766(3q13)$$

$$\text{Control } y = -1.812 + 0.346(10p11.2) + 0.623(14q12.3) + 0.336(3q13)$$

which reduces to the single classification function: $0.542(10p11.2) + 0.561(14q12.3) + 0.430(3q13)$ and from this function an individual could be classified as TS if the observed value of this function for these three sites was more than 4.

In six instances, similarly affected family members of index cases with TS formed part of the study. The sites recurring within these families include, in family 1 (a male and a female): 15q15 in 2/2 family members; family 2 (three females and a male): 3q21 in 4/4 cases and 6q23 in 3/4 cases; family 3 (three males and a female): 17p12 in 3/4 and Yq11–12 in 2/4 cases, respectively; family 4 (three males and female): 17p12 in 4/4 and Yq11–12 in 3/4 cases, respectively; family 5 (a male and two females): 8p23 in 2/3 cases. In family 6, in two females, there was no concordance with regard to any FS. Yq11–12 was expressed in 7/9 males from these family groups. Some of these breaks are not reflected in Table I and II because they do not qualify for inclusion according to the criteria mentioned in the methods section of this paper.

TABLE II. Mean (SD) Percentage of Other Breaks for TS and Control Patients

Site	Tourette patients	Controls	*P* values associated with t-test
3p26[a]	2.42 (2.32)	1.21 (1.32)	0.027
3q13[a]	3.73 (2.44)	1.63 (1.17)	0.000
4q23[a]	0.65 (0.94)	0.25 (0.53)	0.065
4q27[a]	0.69 (1.09)	0.50 (0.72)	0.469
4q34-5	*1.00* (1.26)	0.63 (1.01)	0.256
5q13	1.38 (1.27)	0.67 (0.82)	0.022
5q33-34	2.15 (1.80)	1.42 (1.50)	0.125
8p21	0.62 (0.75)	0.33 (0.56)	0.139
8q11.2	0.35 (1.02)	0.46 (0.98)	0.693
10p13	1.19 (1.30)	1.96 (1.49)	0.583
10p11.2	3.08 (1.72)	1.29 (1.27)	0.000
10q11.2	0.12 (0.33)	0.21 (0.41)	0.381
12p12	0.77 (0.82)	0.75 (0.79)	0.933
12q13[b]	1.23 (1.21)	0.38 (0.65)	0.003
13q34[a]	0.42 (0.81)	0.46 (0.66)	0.867
14q13	3.92 (1.94)	2.00 (1.38)	0.000
15q13-4	0.50 (0.65)	0.67 (0.82)	0.426
18p11.2	1.15 (1.35)	0.96 (1.27)	0.600
18q22	0.96 (1.08)	0.46 (0.88)	0.079
20q11.2	0.73 (0.78)	0.50 (0.72)	0.283
21q22	0.92 (0.80)	0.46 (0.66)	0.030
22q13[b]	0.42 (0.75)	0.08 (0.28)	0.041
Xp22.1	0.96 (1.15)	0.29 (0.55)	0.012
Xq13	2.08 (1.38)	0.96 (1.04)	0.002
Xq24	0.58 (0.76)	0.29 (0.69)	0.172
Xq26	0.62 (0.90)	0.50 (0.72)	0.621
Xq27.3[b]	0.04 (0.20)	0.13 (0.34)	0.280

[a]Breakpoints detected in our study and reported by others, but not listed in CCM92
[b]Breakpoints considered as rare folate-sensitive FS

DISCUSSION

This paper reports increased aphidicolin induced common fragile site (FS) expression associated with Tourette syndrome (TS), as well as the possible existence of discriminatory fragile sites with regard to TS patients. The particular sites found to be indicative of TS during this early study may be altered during subsequent research.

Confirmation of increased aphidicolin-induced FS expression in TS needs to take into account the large number of conditions which may influence chromosome breakage [Craig-Holmes et al., 1987; Chudley et al., 1990; Smeets and Merkx, 1990; Tedeschi et al., 1992], including tissue specificity of FS expression [Morgan et al., 1988; Murano et al., 1989], culture conditions such as ethanol concentration [Kuwano and Kajii, 1987], menstrual stage cycle in females [Furuya et al., 1991], and knowledge of the population background with regard to common aphidicolin-induced FS [Rao et al., 1988]. Although our study could be criticized for not adequately evaluating the role of such variables, the differences between TS and control individuals were consistently different to such a marked degree that it was considered worthwhile to open this avenue of research to the widest possible scrutiny.

A major question arises whether increased expression of aphidicolin-induced fragile sites in individuals with Tourette syndrome could be potentially informative for the molecular analysis of genes involved in the expression of a TS spectrum disorder. The future isolation and cloning of the DNA sequences involved at some of these sites might show that they are polymorphic regions and targeted association studies could potentially be applied to examine their role in TS as well as other neuropsychiatric disorders.

A number of reports correlating behavioral alteration with chromosomal breakage have already been published and includes schizophrenia [DeLisi et al., 1988; Garofalo et al., 1992], the psychopathology found in obligate fra-X female carriers [Freund et al., 1992], and Rett syndrome [Telvi et al., 1994].

To our knowledge, the only published finding relating chromosomal fragility to TS by another author can be found in an article on Huntington disease and childhood-onset Tourette syndrome [Kerbeshian et al., 1991], where the karyotype 46,XY/46,XY,fra(16)(q22) was found in 12% of cells. The authors stated that the clinical significance of FS in phenotypically normal individuals is unknown.

In addition to being considered genetically active areas [Hecht and Hecht, 1991: Yunis et al., 1987: Hecht et al., 1988: Austin et al., 1992], FS may represent regions of DNA repeat sequences [Sutherland et al., 1985]. Organized repetitive DNA sequences in the genome are considered to bear a relationship to a highly conserved chromatin folding code [Vogt, 1990] and may predispose these areas to selective forces such as environmentally induced breakage.

Individuals expressing the rare folate-sensitive sites FRAXA and FRAXE have unstable expanded CCG repeats and methylation of adjacent CpG islands. An explanation has been proposed according to which GCC and CGG repeat sequences lead to delayed replication because they form unusual DNA structures that present a block for the replication apparatus [Knight et al., 1993]. Chromatin is subsequently rendered fragile through late replication following failure to erase an imprinted X-inactivation signal in these instances [Laird et al., 1987].

In addition, the molecular basis of FRA16A, another autosomally located, rare folate sensitive fragile site, was found to be expansion of a normally polymorphic p(CCG)n repeat [Richards et al., 1994]. It is not clear whether one is allowed to extrapolate from these findings concerning rare fragile sites. Molecular analysis of a 4.5 kB fragment containing 6 of 13 aphidicolin-induced breakpoints at constitutive fragile site 3p14.2 failed to identify traditional motifs, such as a trinucleotide repeat sequence to explain fragility [Paradee et al., 1994]. However, in a cell line with a reciprocal translocation between human chromosome 3 (with breakpoint at 3p14.2) and a hamster chromosome, the fragile site was expressed on both derivative chromosomes, suggesting in this instance that the fragile site represents a repeated sequence [Glover and Stein, 1988].

If multiple genes, acting either independently (genetic heterogeneity), additively (polygenic inheritance), or epistatically, are required for expression of a neurobehavioral spectrum phenotype, this will create obsta-

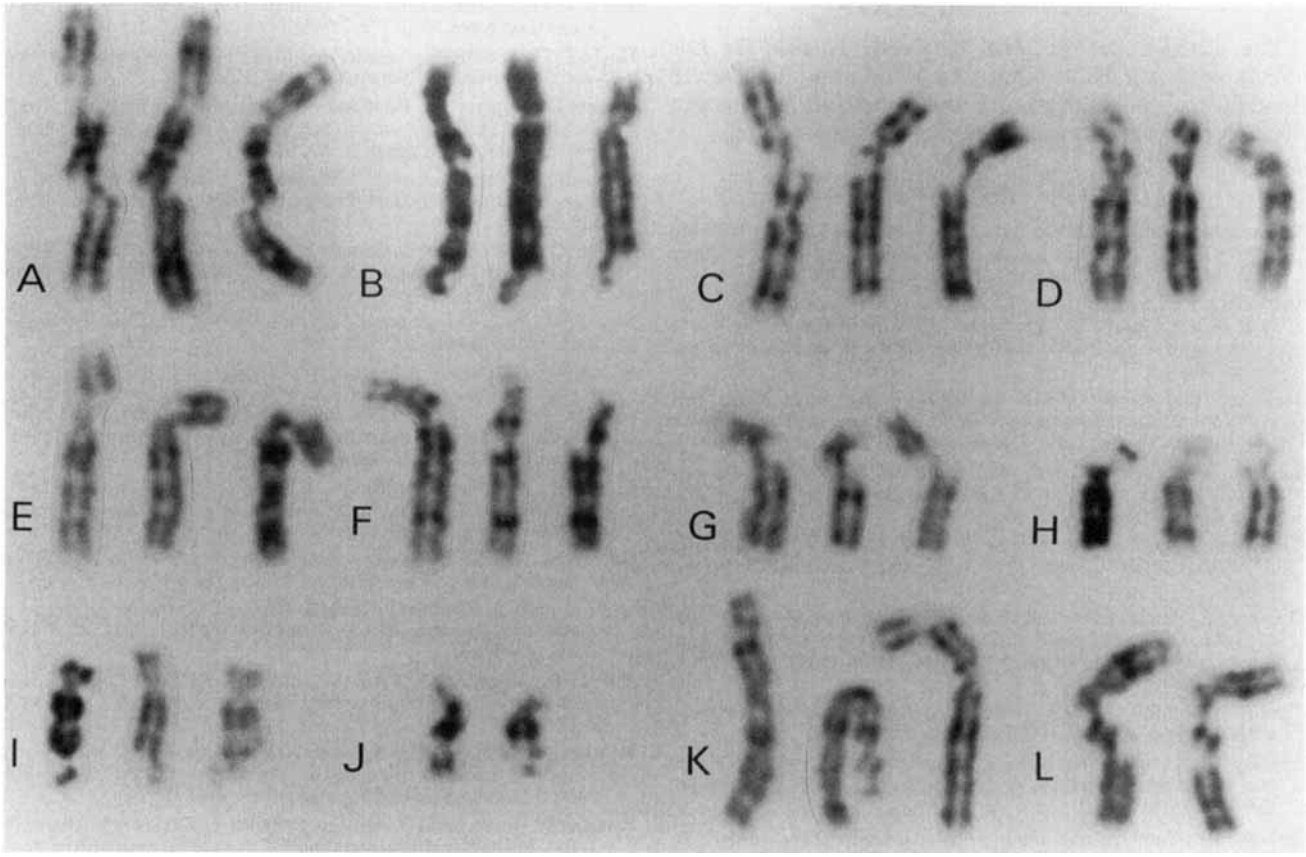

Fig. 1. Examples of some category II breaks in TS patients: **A**, 3q13*; **B**, 4q33-q34; **C**, 5q13; **D**, 10p13; **E**, 10p11.2; **F**, 14q13; **G**, 15q13-q14; **H**, 18p11.2-p11.3; **I**, 18q22; **J**, 21q22; **K**, Xp22.1; **L**, Xq13.

cles for linkage detection of a major gene by the standard lod score method [Crowe, 1993]. Nonparametric linkage analyses, such as the affected sib-pair method, which is useful for the analysis of multifactorial disorders, and which was suggested as the next step in TS-gene linkage at the Tenth Genetic Workshop on Tourette Syndrome held during August 1994 in Toronto (personal communication, J Weber) is less powerful to detect linkage and cannot satisfactorily address a situation where multiple genes may be operative, which is what our fragility studies could suggest.

For TS, a dual situation may exist, according to which various associated phenotypes may arise through modified gene activity at fragile sites ("the component factors of multidimensional phenotypes") [Cloninger, 1994], but the primary phenotype for which the TS gene is responsible may actually be chromosomal instability.

A heritability estimate of 0.88 for aphidicolin-inducible common FS site expression in a twin study indicated that fragility could be considered to represent the secondary expression of a more fundamental mechanism operative within the genome [Austin et al., 1992]. Similarly, a basic genetic mechanism was proposed for the concurrent expression of both autosomal FS and fragile-X sites in individuals from three families with the fra-X syndrome [Barletta et al., 1991]. This concept was also discussed by other groups [Amarose et al., 1987; Smeets and Ares, 1990].

Since individuals with TS are usually phenotypically normal, an association between FS and neurobehavioral characteristics (rather than with dysmorphic or disease-related phenomena) may easily have been overlooked during earlier population based surveys of common FS. The quotation by Roger Kurlan that ". . . there is at least a little bit of TS in us all" [Kurlan, 1993] seems entirely appropriate in view of the population frequency of expression of common FS and the proposed role of some change at these sites being associated with neurobehavioral alteration.

In conclusion, if differential expression of certain fragile sites can be confirmed to be associated with variant behavior, and these FS can also be demonstrated to show a consistent relationship with certain classes of repeat sequences, such sites may be important with regard to both normal evolutionary processes [King, 1994], as well as having the potential to be involved in potentially deleterious dynamic mutations.

As indicated, these findings do not rule out the need to search for a major TS gene, which may however not be as strongly linked to any of the behavioral phenotypical features as to the chromosomal phenotype which is described in this paper.

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